RAPID NUCLEIC ACID ISOLATION METHOD
AND COMPOSITIONS THEREFORE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is based on Serial No.
60/489,017 filed on July 21, 2003.

BACKGROUND OF THE INVENTION

Living cells contain various types of RNA,
although three particular types make up the
predominant proportion, namely ribosomal RNA (rRNA,
approximately 80 percent of all RNAs in a cell),
transfer RNA (tRNA, approximately 5 percent of all
RNAs in a cell) and messenger RNA (mRNA,
approximately 2-4 percent of all RNAs in a cell). A
variety of other RNA types are known, including
heterogeneous nuclear RNA (hnRNA), small cytoplasmic
RNA (scRNA), small nuclear RNA (snRNA), small
nucleolar RNA (snoRNA), regulatory RNAs, guide RNAs,
telomerase RNA and small regulatory RNAs. Isolation
of total RNA is typically designed to isolate
substantially all of the three predominate types of
RNA, that is rRNA, tRNA and mRNA, although minor
amounts of the remaining RNA types can be present in
the total RNA isolation.

High quality RNA is a prerequisite for
various gene expression assays such as reverse
transcriptase (RT) PCR, primer extension and Southern
or Northern blotting techniques, as well as for
preparation of RNA probes for these and similar
techniques. In addition novel drug therapies require
examining high quality RNA from many species of
microbial pathogens. Methods of extracting RNA are

known, but those methods have various undesirable characteristics.

Guanidinium thiocyanate and guanidinium chloride are known as effective protein denaturants, and the chloride salt has been employed as a deproteinization agent for isolating total RNA, see Cox, R. A.: Methods in Enzymology, 12(B): 120-129 (1968). In the Cox procedure, a suspension of ribosomes in a buffer is added to 6 molar guanidinium chloride. The RNA is precipitated by adding alcohol to the solution and the precipitate is recovered by centrifugation. At this point, the precipitate is not free of RNase (a contaminating enzyme that causes RNA degradation) and is purified by dissolution in 4 molar guanidinium chloride and re-precipitation by adding ethanol. The precipitate is again separated by centrifugation. If further purification is necessary, the precipitate is redissolved in guanidinium chloride and re-precipitated with ethanol. This procedure takes approximately two days to complete.

Another method for isolating total RNA is disclosed in Chirgwin, J. M. et al: Biochem, 18:5294-5299 (1979). In that procedure, RNA-containing tissue is homogenized in a solution containing guanidinium thiocyanate, sodium citrate and 2-mercaptoethanol, with the solution pH adjusted to 7 by adding sodium hydroxide. The homogenate is then centrifuged and the supernatant decanted and mixed with acetic acid (to lower the pH value to 5) and absolute ethanol. Overnight storage at -20 °C precipitates the RNA and it is recovered in pellet form after centrifugation. The pellet is redissolved in a buffered guanidine hydrochloride solution and

reprecipitated by adding acetic acid and ethanol.
The last step is repeated and the isolated RNA is
recovered in pellet form.

Alternatively, the RNA can be separated
from the guanidinium thiocyanate homogenate by
ultracentrifugation through a dense cushion of cesium
chloride. This method is disadvantageous in that it
can take up to 2 days to obtain results and it can
require the use of expensive ultracentrifugation
equipment which reduces the total amount of RNA
recoverable because of the limited number of samples
that can be simultaneously processed.

Yet another RNA-isolating procedure is
shown in Feramisco, J. R. et al: Molecular Cloning,
194-195, Cold Spring Harbor Laboratory, Cold Spring
Harbor, N.Y. (1982), in which RNA-containing tissue
is homogenized in a solution of 4M guanidinium
isothiocyanate, 20% Sarkosyl™ (sodium lauryl
sarkosinate) and 2-mercaptoethanol. An equal volume
of heated phenol (approximately 60 °C) is added to
the homogenate along with a sodium acetate solution
of pH 5.2. Then an equal volume of chloroform is
added and the mixture is cooled and centrifuged. The
aqueous phase is recovered and reextracted with
phenol and/or chloroform about seven more times
before the final product is recovered. This
procedure is slow and cumbersome in that multiple
extractions are required and it takes approximately
two days to recover the available RNA.

In U.S. Patent No. 4,843,155, phenol and
guanidine procedures were combined, resulting in a
method of total RNA isolation that can be completed
in 3 hours. The method of that patent was further
improved upon in U.S. Patent No. 5,346,994, which

allows for completion of the RNA isolation in about 1
hour. Those methods involve the use of a mono-phasic
solution of phenol and guanidine isothiocyanate,
commercially available as the reagent Trizol®
(Invitrogen Corp., Carlsbad, CA).

It has been found that total RNA samples
extracted from clinical isolates using Trizol® or hot
phenol methods can experience unacceptable levels of
high molecular weight DNA contamination, as
determined by ethidium bromide gel electrophoresis.
That DNA contamination compromises the purity of the
isolated RNA, making subsequent use of the RNA
difficult or impossible. Unexpectedly, the methods
and compositions of the present invention permit the
easy preparation of highly pure RNA samples from
clinical isolates with a minimum amount of
contaminating genomic DNA. In addition, the methods
and compositions of the present invention provide for
higher yields and higher purity of total RNA
isolated, when compared to other methods.

BREIF SUMMARY OF THE INVENTION

In one aspect, the present invention
contemplates a method of isolating RNA from a
biological specimen by first contacting the
biological specimen with an admixture of (i) a mono-
phasic solution of phenol and guanidine
isothiocyanate, and (ii) a lysis buffer, under
conditions and for a time appropriate to form a
homogenate. Next, the homogenate is admixed with a
water-immiscible organic solvent under conditions and
for a time appropriate to form an aqueous phase and
an organic phase. The aqueous phase is then
contacted with a C_1-C_4 lower alcohol under conditions

and for a time to form a precipitated RNA. The precipitated RNA is then recovered, as by centrifugation and decanting of the aqueous phase.

In another aspect, the present invention contemplates a method of isolating RNA from a biological specimen by contacting the biological specimen with (i) a lysis buffer, and (ii) a mono-phasic solution of phenol and guanidine isothiocyanate, under conditions and for a time appropriate to form a homogenate. Next, the homogenate is admixed with a water-immiscible organic solvent under conditions and for a time appropriate to form an aqueous phase and an organic phase. The aqueous phase is then contacted with a C_1-C_4 lower alcohol under conditions and for a time to form a precipitated RNA. The precipitated RNA is then recovered, as by centrifugation and decanting of the aqueous phase.

In yet another aspect, the present invention contemplates a method of isolating total RNA from a biological specimen by first contacting the biological specimen with an admixture of (i) a mono-phasic solution of phenol and guanidine isothiocyanate and (ii) a lysis buffer under conditions and for a time appropriate to form a homogenate. Next, the homogenate is admixed with a water-immiscible organic solvent under conditions and for a time appropriate to form an aqueous phase and an organic phase. The aqueous phase is then contacted with a C_1-C_4 lower alcohol under conditions and for a time to form a precipitated total RNA. The precipitated total RNA is then recovered, as by centrifugation and decanting of the aqueous phase.

In a further aspect, the present invention contemplates a method of isolating total RNA from a biological specimen by contacting the biological specimen with (i) a lysis buffer, and (ii) a mono-phasic solution of phenol and guanidine isothiocyanate, under conditions and for a time appropriate to form a homogenate. Next, the homogenate is admixed with a water-immiscible organic solvent under conditions and for a time appropriate to form an aqueous phase and an organic phase. The aqueous phase is then contacted with a C_1-C_4 lower alcohol under conditions and for a time to form a precipitated total RNA. The precipitated total RNA is then recovered, as by centrifugation and decanting of the aqueous phase.

In a still further aspect, the present invention contemplates a method of isolating total RNA from a Gram-positive bacterium by contacting the Gram-positive bacterium with (i) a lysis buffer, and (ii) a mono-phasic solution of phenol and guanidine isothiocyanate, under conditions and for a time appropriate to form a homogenate. Next, the homogenate is admixed with a water-immiscible organic solvent under conditions and for a time appropriate to form an aqueous phase and an organic phase. The aqueous phase is then contacted with a C_1-C_4 lower alcohol under conditions and for a time to form a precipitated total RNA. The precipitated total RNA is then recovered, as by centrifugation and decanting of the aqueous phase.

Preferably, the biological specimen is a clinical isolate of a microorganism. The microorganism can be a bacterium, a virus, a fungus, or a combination thereof. The biological sample can

be of human, animal, plant or microbial origin.
Preferred bacteria are Gram-positive bacteria.

The lysis buffer comprises a chelating
agent and a dispersing agent. In preferred
embodiments, the chelating agent is EDTA. In other
preferred embodiments, the chelating agent is EGTA.
In an especially preferred embodiment, the chelating
agent is a combination of EDTA and EGTA.

In preferred embodiments, the dispersing
agent is a detergent. In another preferred
embodiment, the dispersing agent is a surfactant. In
an especially preferred embodiment, the surfactant is
N-lauroylsarcosine. In another especially preferred
embodiment, the surfactant is sodium lauryl sulfate.

Preferred water-immiscible organic solvents
include chloroform and carbon tetrachloride.
Preferred C_1-C_4 lower alcohols include ethanol,
methanol and isopropyl alcohol.

In another aspect, the invention
contemplates a composition of matter comprising an
admixture of (i) a mono-phasic solution of phenol and
guanidine isothiocyanate and (ii) a lysis buffer.
This composition of matter is useful for isolating
RNA or total RNA from a biological specimen.

In still another aspect, the invention
contemplates a kit comprising an admixture of (i) a
mono-phasic solution of phenol and guanidine
isothiocyanate and (ii) a lysis buffer.

In a further aspect, the invention
contemplates a kit comprising (1) a mono-phasic
solution of phenol and guanidine isothiocyanate and
(2) a lysis buffer.

Preferably, the kit contains instructions for isolating RNA or total RNA from a biological sample.

The lysis buffer comprises a chelating agent and a dispersing agent. In preferred embodiments, the chelating agent is EDTA. In other preferred embodiments, the chelating agent is EGTA. In an especially preferred embodiment, the chelating agent is a combination of EDTA and EGTA. In preferred embodiments, the dispersing agent is a detergent. In other preferred embodiments, the dispersing agent is a surfactant. In an especially preferred embodiment, the surfactant is N-lauroylsarcosine. In another especially preferred embodiment, the surfactant is sodium lauryl sulfate.

The present method has a number of benefits and advantages for the isolation of RNA and/or total RNA from biological specimens, including clinical microorganisms, without the use of hazardous materials as compared those methods that require the use of heated phenol, a dangerous corrosive material. The method of the invention is rapid and reproducible, yet provides yields comparable to other methods of nucleic acid isolation. The present method is economical and environmentally friendly, as it does not require the use of heated phenol or other components that are either costly or hazardous, and requires the use of less Trizol® than is currently necessary. The present method provides for preparation of RNA and/or total RNA samples that are substantially free from DNA contamination, as visualized by gel electrophoresis. The present method is suitable for extraction of RNA or total RNA from fresh clinical isolates of different species of

microorganisms, such as viruses, bacteria, and fungi, including clinical isolates of *Escherichia coli*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is shown on two sheets as Fig. 1A and Fig. 1B, in which Fig. 1A shows a photograph of a 1 percent agarose gel stained with ethidium bromide. The primary start sites of transcription were mapped using primer extension analysis as indicated by the arrow, using techniques set forth in the Examples elsewhere herein. The intensities of the bands were quantified using a Storm Molecular Imager with ImageQuant™ software (Molecular Dynamics Inc., Sunny, CA, USA) and normalization of data was obtained using the 16S rRNA bands. Extension products were visualized by exposing the gel to a storage phosphor (Eastman Kodak Co., Rochester, NY, USA) for 2 days. The bla_{ACT-1} and bla_{MIR-1} sequencing ladders are represented on the left and right sides, respectively. The bands used to map the start site of transcription represent the amount of *ampC* RNA expression from each strain. These bands were quantified and normalized using the 16S rRNA bands. Lane 1 depicts the WT*ampC* gene of *Enterobacter cloacae* strain 55. Lane 2 depicts the bla_{ACT-1} gene of *Klebsiella pneumoniae* strain 255. Lane 3 depicts the bla_{MIR-1} gene of *Klebsiella pneumoniae* strain 96D. Lane 4 depicts the AR*ampC* gene of *Enterobacter cloacae* strain V 204. Fig. 1B shows a bar graph representing *ampC* expression of bla_{ACT-1}, bla_{MIR-1} and

DRampC relative to WTampC. Each value represents the
mean of three experiments. Error bars represent the
standard deviation.

Fig. 2 shows a photograph of three separate
1 percent agarose gel stained with ethidium bromide.
Lanes 1-3 of the first gel (on the left) depict RNA
samples isolated using the hot phenol method, as set
forth in Example 2 elsewhere herein. Lanes 4-6 of
the first gel depict RNA samples isolated using a
Qiagen™ kit based on Trizol®, according to the
manufacturer's instructions. The presence of high
molecular weight DNA contamination can be seen in all
six lanes. Lanes 2-5 of the middle gel show the
results of RT-PCR of a Pseudomonas aeruginosa OprM
gene. Lanes 2-5 of the third gel (on the right) show
the results of RT-PCR of a Pseudomonas aeruginosa
MexC gene. Lane 1 of the middle and third gels shows
a 100 base pair molecular weight ladder.

Fig. 3 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lanes 2-
11 show the results of RT-PCR of Enterobacter cloacae
ribosomal RNA using RNA samples isolated using a
method of the invention. Lane 1 of the gel shows a
100 base pare molecular weight ladder.

Fig. 4 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lanes 2-5
of the gel show the results of RT-PCR of a
Pseudomonas aeruginosa efflux pump associated gene
mexA using RNA samples isolated using a method of the
invention. Lane 1 of the gel shows a 100 base pair
molecular weight ladder.

Fig. 5 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lanes 2-6
of the gel show a linearity curve of RT-PCR of the

ampC gene from *Serratia marcescens* using RNA samples
isolated using a method of the invention. Lane 1 of
the gel shows a 100 base pair molecular weight
ladder.

Fig. 6 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lanes 1
and 2 depict RNA samples from clinical isolates of
Citrobacter freundii. Lanes 3 and 4 depict RNA
samples from clinical isolates of *Enterobacter
cloacae*. Lanes 5 and 6 depict RNA samples from
clinical isolates of *Escherichia coli*. All RNA
samples were isolated using a method of the
invention.

Fig. 7 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lanes 2-5
depict a linearity curve of a *Pseudomonas aeruginosa*
oprD gene. Lane 1 shows a 100 base pair molecular
weight ladder. Determination of a linearity curve is
part of the analysis for gene expression when the
level of expression of several strains is compared.
RT-PCR was carried out according to methods set forth
in the Examples elsewhere herein, and samples were
removed at the end of each cycle and resolved in the
gel shown here in order to determine how many cycles
the reaction should be allowed to proceed that
permits optimal expression of the gene.

Fig. 8 shows a photograph of a 1 percent
agarose gel stained with ethidium bromide. Lane A
shows total RNA isolated from *Enterobacter cloacae*
using a method of the invention. Lane B shows total
RNA isolated from *Enterobacter cloacae* using Trizol®,
according to the manufacturer's instructions except
that a homogenizer was not used. Lane C is a 100
base pair molecular weight ladder.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention contemplates a method of isolating RNA from a biological specimen by first contacting the biological specimen with an admixture of (i) a mono-phasic (one phase) solution of phenol and guanidine isothiocyanate and (ii) a lysis buffer under conditions and for a time appropriate to form a homogenate. Alternatively, the RNA can be isolated from a biological specimen by contacting the biological sample first with (i) a lysis buffer, followed by (ii) a mono-phasic solution of phenol and guanidine isothiocyanate.

An admixture of (i) a mono-phasic solution of phenol and guanidine isothiocyanate and (ii) a lysis buffer is prepared according to techniques well known to workers of ordinary skill in the art. For example, prepared solutions of (i) a mono-phasic solution of phenol and guanidine isothiocyanate and (ii) a lysis buffer are manually mixed together for a time sufficient to form an admixture. Alternatively, those prepared solutions can be mixed together mechanically, such as by use of a vortex mixer (for laboratory scale mixing) or mixers, shakers, rollers and the like for industrial scale mixing. The relative proportions of mono-phasic phenol and guanidine isothiocyanate solution to lysis buffer is typically about 10:1 to about 1:1, and more usually about 5:1 to about 4:1, by volume.

The resulting admixture is then contacted with a biological specimen according to techniques well known to those of ordinary skill in the art. For example, a bacterial culture can be grown in a

liquid culture medium, and a bacterial pellet
prepared by ultracentrifugation. The resulting
bacterial pellet can be resuspended in the admixture
of the invention. Exemplary reaction times and
conditions for formation of the resulting homogenate
are set forth elsewhere herein. Alternatively, a
fresh clinical isolate can be directly introduced
into the admixture of the invention to form a
homogenate.

In alternative embodiments, the method of
the invention involves contacting a biological
specimen first with a lysis buffer. The biological
specimen can remain in contact with the lysis buffer
for a time, under reaction times and conditions set
forth elsewhere herein, or a mono-phasic solution of
phenol and guanidine isothiocyanate can be added
immediately after the lysis buffer. Once the latter
solution is added, the homogenate is formed under
reaction times and conditions set forth elsewhere
herein.

In preferred embodiments, the methods of
the invention are used for the isolation of total
RNA. "Isolation of total RNA" does not necessarily
mean that all RNA found in a cell is isolated, but
only that a majority of the predominant RNA types
have been isolated and are available for further use.
For example, total RNA will typically contain
substantial amounts of mRNA as well as rRNA, tRNA and
other types of RNA, although not necessarily in the
same percentages as those RNA types naturally exist
in the biological specimen. When total RNA is
isolated from eukaryotic cells, the mRNA can be
further isolated from the total RNA by use of, for
example, a poly(dT) column, which column selectively

binds the poly(A) tail found on most eukaryotic mRNA, according to techniques well known to those of ordinary skill in the art.

The appropriate reaction conditions and times are well known to workers of ordinary skill in the art. For example, the reaction conditions can be performed at high temperatures, such as from about 90 to about 100° C, preferably about 95° C, in order to facilitate cell lysis and denaturation of protein contaminants. In such instances, the time required for cell lysis is usually brief, from about 2 to about 10 minutes, preferably about 4 minutes.

Alternatively, the reaction conditions can be performed at around normal human body temperatures, of about 37° C, but the time for cell lysis can be longer, requiring from about 10 to about 30 minutes of incubation time. Generally, as the reaction temperatures decrease, the time required for adequate cell lysis increases. Moreover, the reaction times and conditions can vary depending upon the sample size. The reaction times and conditions exemplified herein are directed to laboratory scale assays, as opposed to industrial scale assays. Nevertheless, scale up of the methods of the invention is well within the capabilities of workers of ordinary skill in the art.

Further incubation at about human body temperature, or at room temperature (from about 20 to about 24 °C) can be carried out, for times ranging from about 2 to about 10 minutes, preferably about 5 minutes.

Preferably, the biological specimen is a clinical isolate of a microorganism. These clinical

isolates can be swabs, mucous samples, exudates, aspirates, biopsy samples, and the like.

The microorganism can be a bacterium, a virus, a fungus, or a combination thereof. Clinical isolates are often assayed for the presence of a microbial contaminant, such as a microorganism that is causing an infection. Rapid identification of the causative microorganism can involve the preparation of RNA samples for subsequent assays, such as RT-PCR or Northern blot analysis. Similarly, clinical isolates can be useful for forensic analysis, such as morbidity and mortality studies on infectious microorganisms.

It is to be understood, however, that the biological specimen can be of human, animal, plant or microbial origin. The invention is not limited to human infectious agents, and is adaptable to biological specimens and samples from any source that is known to contain, or may contain, an RNA of interest.

The mono-phasic solution of phenol and guanidine isothiocyanate can be prepared according to methods well known in the art, and as set forth in U.S. Patent No. 5,346,994, whose disclosures are incorporated in their entirety herein. A mono-phasic solution of phenol and guanidine isothiocyanate is commercially available as Trizol® (Invitrogen Corp., Carlsbad, CA).

The lysis buffer comprises a chelating agent and a dispersing agent. However, other components can be present, such as buffering agents to provide an appropriate pH value for the resulting solution, and salts, sugars and the like to provide appropriate osmolality. The pH value of the lysis

buffer can be from about 4.0 to about 6.0, preferably
from about 4.8 to about 5.2, and most preferably
about 5.0.

Without wishing to be bound by theory, it
is believed that the chelating agent is present to
chelate or sequester certain ions that can be present
in the biological sample. For example, biological
samples often contain magnesium or manganese ions
that can be chelated with chelating agents that are
well known to workers of ordinary skill in the art.

The term "chelating agent" as used herein refers
to any organic or inorganic compound that will bind
to a metal ion having a valence greater than one.
Chelating agents include, but are not limited to,
organic chelating agents such as ethylenediamene-
tetraacetic acid (EDTA), triethylenetetramine
dihydrochloride (TRIEN), ethylene glycol
bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid
(EGTA), diethylenetriamine-pentaacetic acid (DPTA),
and triethylenetetramine-hexaacetic acid (TTG),
deferoxamine, Dimercaprol, edetate calcium disodium,
zinc citrate, penicillamine succimer and Editronate
or any other chelating agent that can chelate
divalent ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, and Zn^{2+}.

Such chelating agents can also be selected
from the group consisting of aminocarboxylates,
aminophosphonates, polyfunctionally-substituted
aromatic chelating agents and mixtures therein, all
as hereinafter defined.

Aminocarboxylates useful as chelating
agents include ethylenediaminetetracetates,
N-hydroxyethylethylenediaminetriacetates, nitrilo-
tri-acetates, ethylenediaminetetraproprionates,
triethylenetetraaminehexacetates,

diethylenetriaminepentaacetates, and ethanoldi-
glycines, alkali metal, ammonium, and substituted
ammonium salts therein and mixtures therein.

Aminophosphonates are also suitable for use
as chelating agents in the compositions of the
invention, and include ethylenediaminetetrakis-
(methylenephosphonates) known commercially as DEQUEST®
(available from Solutia Inc., St. Louis, Missouri).
Preferably, these aminophosphonates do not contain
alkyl or alkenyl groups with more than about 6 carbon
atoms.

Polyfunctionally-substituted aromatic
chelating agents are also useful in the compositions
herein. See U.S. Pat. No. 3,812,044, issued May 21,
1974, to Connor et al. Preferred compounds of this
type in acid form are dihydroxydisulfobenzenes such
as 1,2-dihydroxy-3,5-disulfobenzene.

A biodegradable chelating agent is
ethylenediaminedisuccinate ("EDDS"), especially the
[S,S] isomer as described in U.S. Pat. No. 4,704,233,
Nov. 3, 1987, to Hartman and Perkins.

The compositions herein can also contain
water-soluble methyl glycine diacetic acid (MGDA)
salts (or acid form) as a chelating agent. Similarly,
the so-called "weak" builders such as citrate can
also be used as chelating agents.

In preferred embodiments, the chelating
agent is EDTA. In other preferred embodiments, the
chelating agent is EGTA. In an especially preferred
embodiment, the chelating agent is a combination of
EDTA and EGTA. Workers of ordinary skill in the art
can readily determine other chelating agents, or
other combinations of chelating agents, useful in the
present invention.

Without wishing to be bound by theory, the dispersing agent is believed to be present to disrupt or solubilize various components of the biological specimen. For example, a dispersing agent can disrupt a cell wall and/or cell membrane or solubilize various proteins, lipoproteins and/or lipids often found in biological samples. In some instances, preferred dispersing agents perform these functions because of surface active, or surfactant, properties of the detergents or surfactants used. However, it is not necessary for the invention that the dispersing agent has surfactant properties. A surfactant or surface-active agent can be a compound in one of three categories: a detergent, a wetting agent or an emulsifier. See, R. J. Lewis, Jr., Hawley's, Condensed Chemical Dictionary, 12th ed., Van Nostrand Reinhold Company, New York, 1993, page 1108. Detergents, with and without surface active properties, are well known in the art.

Natural and synthetic surfactants that are useful in the present invention include those classified as nonionic, anionic, cationic, zwitterionic or amphoteric. Non-limiting examples of such surfactants useful in the present invention, either alone or in combination, as classified by chemical type, include the following:

alcohols (such as refined soybean sterol, a nonionic surfactant);

alkanolamides (such as coconut diethanolamide, 1:1 type, a nonionic surfactant); alkanolamines (such as ethoxylated coconut monoethanolamine, a nonionic surfactant);

alkylaryl sulfonates (such as sodium alkyl aryl sulfonate, an anionic surfactant);

alkylaryl sulfonic acids (such as linear dodecyl benzene sulfonic acid, an anionic surfactant);

amine acetates (such as acetic acid salts of the n-alkyl amines, cationic surfactants);

amine oxides (such as coco amido propyl dimethyl amine oxide, cetyl dimethyl amine oxide, lauryl dimethyl amine oxide, myristyl cetyl dimethyl amine oxide, and myristyl dimethyl amine oxide, all noninonic surfactants);

amines (such as primary alkylamines and cocamidopropyl dimethylamine, both cationic surfactants); sulfonated amines and amides (such as sulfated fatty amide, amide sulfonate, and isopropylamine sulfonate, all anionic surfactants);

betaine derivatives (such as cocamidopropyl betaine, oleyl betaine, coconut alkyl dimethyl ammonium betaine, and lauramidopropyl betaine, all amphoteric surfactants);

block polymers (such as block copolymers of propylene oxide and ethylene oxide, nonionic surfactants);

carboxylated alcohol or alkylphenol ethoxylates (such as carboxylated alkyl polyether, alkyl polyethylene glycol ether acetic acid, alkylether carboxylic acid, and carboxylated alcohol ethoxylates, all anionic surfactants);

diphenyl sulfonate derivatives (such as disodium mono- and di-dodecyl diphenyl oxide disulfonate, sodium hexyl diphenyloxide disulfonate, dodecyl diphenyloxide disulfonic acid, sodium dodecyl diphenyl ether disulfonate, and disodium alkyl diphenyloxide sulfonate, all anionic surfactants);

ethoxylated alcohols (such as
polyoxyethylene (4) lauryl ether, polyoxyethylene (2)
cetyl ether, polyoxyethylene (10) cetyl ether,
polyoxyethylene (20) stearyl ether, ethoxylated oleyl
alcohol, fatty alcohol polyglycol ether, ethoxylated
lauryl alcohol, ethoxylated aliphatic alcohol, and
alkyl polyether, all nonionic surfactants);
ethoxylated alkylphenols (such as alkylphenol
polyethylene glycol ether, nonylphenoxy
polyethoxyethanol, octylphenol ethoxylate,
nonylphenol ethoxylate, and
octylphenoxypolyethoxyethanol, all nonionic
surfactants);

ethoxylated amines and/or amides (such as
fatty amine polyglycol ethers [both nonionic and
cationic surfactants], polyoxyethylated (30) oleyl
amine and polyoxyethylated (5) tallow amine
[cationic surfactants], ethyloxylate coco amine and
ethoxylated tallow amine [nonionic surfactants];

ethoxylated fatty acids (such as ethylene
glycol monostearate, polyethylene glycol (400)
monoricinoleate, PEG-600 monolaurate, PEG-600
dioleate, oleic acid polyglycol ester, diethylene
glycol monostearate, all nonionic surfactants);

ethoxylated fatty esters and oils (such as
polyoxyethylene fatty glyceride, polyoxyethylene
polyol fatty acid ester, polyoxyethylene 20 sorbitan
monolaurate, PEG-2 castor oil, and ethoxylated castor
oil, all nonionic surfactants);

fatty esters (such as sodium stearoyl
lactylate, calcium stearoyl-2-lactylate [both anionic
surfactants], and butyl oleate [a nonionic
surfactant]);

fluorocarbon-based surfactants (such as
potassium fluorinated alkyl carboxylates, ammonium
perfluoroalkyl sulfonates, and potassium
perfluoroalkyl sulfonates [all anionic surfactants],
fluorinated alkyl amphoteric mixture [an amphoteric
surfactant], and fluorinated alkyl quaternary
ammonium iodides [a cationic surfactant]);

glycerol esters (such as glycerol
monolaurate, glycerol mono/dioleate, propylene glycol
dicaprylate/dicaprate, decaglycerol tetracocate,
triglycerol monostearate, polyglyceryl ester, and
sodium stearyl lactylate, all nonionic surfactants);

glycol esters (such as PEG 400 ditallate
and PEG 200 dilaurate, nonionic surfactants);
heterocyclic type products (such as DL-pyrrolidone
carboxylic acid salt of N^2cocoyl-L-arginine ethyl
ester, a cationic surfactant);

imidazolines and imidazoline derivatives
(such as lauric acid imidazoline and
amidoimidazoline) [both cationic surfactants], and
monocarboxylic coconut-based imidazoline and methyl
alkyl imidazoline sodium salt [both amphoteric
surfactants];

isethionates (such as sodium cocoyl
isethionate, an anionic surfactant);

lanolin-based derivatives (such as lanolin-
derived extracts of sterols and sterol esters
[nonionic surfactants] and lanolin fatty acids
[anionic or nonionic surfactants];

lecithin and lecithin derivatives (such as
lecithin, hydroxylated lecithin, and soy phosphatides
[amphoteric surfactants];

lignin and lignin derivatives (such as
calcium lignosulfonate, an anionic surfactant);

monoglycerides and derivatives (such as gylceryl moncaprylate and glyceryl monolaurate, both nonionic surfactants);

olefin sulfonates (AOS) (such as sodium alpha olefin sulfonate, an anionic surfactant); phosphate esters (such as polyoxyethylene phenyl ester phosphate, aromatic phosphate acid ester, mono- and dialkyl acid phosphate, and alkyl ether phosphate ester (free acid or potassium salt), all anionic surfactants);

phosphorous organic derivatives (such as phosphated mono- and diglycerides, phosphated nonylphenoxy ethanol, phosphated glycol ester, and phosphated linear alcohol ethoxylate, all anionic surfactants); polyethylene glycols;

polymeric [polysaccharide, acrylic acid, acrylamide] (such as C_9-C_{11} alkyl polysaccharide ether and alkyl polyglycosides [nonionic surfactants], and crosslinked hydrophobically-modified polyacrylic acid polymer [anionic surfactants]);

propoxylated and ethoxylated fatty acids, alcohols or alkyl phenols (such as alkoxylated alcohol, ethyoxylated and propoxylated lauryl alcohol, and linear alcohol ethoxylate, all nonionic surfactants); protein-based surfactants (such as TEA-lauroyl collagen amino acids and TEA-abietoyl-hydrolyzed animal protein [anionic surfactants], and POE (25) glycerin mono-pyroglutamic monoisostearic diester [a nonionic surfactant]);

quaternary surfactants (such as trimethyl cetyl quaternary ammonium chloride, polyoxyalkylene amine quaternary and dimethyl dicoco quaternary ammonium chloride, all cationic surfactants); sarcosine derivatives (such as sodium-N-lauryl

sarcosinate, cocoyl sarcosine, and lauroyl sarcosine, all anionic surfactants);

silicone-based surfactants (such as polysiloxane polyether copolymers and silicone glycol copolymers, nonionic surfactants);

soaps (such as potassium coconut oil soap and potassium vegetable oil soap, anionic surfactants);

sodium isethionate (such as sodium salt of cocoyl isethionate in stearic acid, an anionic surfactant);

sorbitan derivatives [such as POE (20) sorbitan monolaurate, sorbitan monooleate, and POE (20) sorbitan monooleate, all nonionic surfactants];

sucrose and glucose esters and derivatives (such as sucrose distearate, ethoxylated (120) methyl glucoside dioleate, and alkylpolyglucoside, all nonionic surfactants);

sulfates and sulfonates of oils and fatty acids (such as sulfated castor oil, sulfated synthetic sperm oil, and sulfonated oleic acid, all anionic surfactants);

sulfates and sulfonates of ethoxylated alkylphenols (such as sodium or ammonium salts of sulfated polyethoxynonylphenol and sodium salt of alkylaryl polyether sulfonate, all anionic surfactants); sulfates of alcohols (such as sodium lauryl sulfate and ammonium hexyl sulfate, anionic surfactants);

sulfates of ethoxylated alcohols; sulfates of fatty esters (such as ammonium alcohol ether sulfate, ammonium laureth sulfate, and sodium alcohol ether sulfate, all anionic surfactants);

sulfates of fatty esters (such as sulfated fatty acid ester, sulfated butyl tallate, and fatty glycerin sulfate, all anionic surfactants);

sulfonates of dodecyl and tridecylbenzenes (such as sodium salt of linear alkybenzene sulfonic acid, sodium dodecylbenzene sulfonate, and sodium tridecylbenzene sulfonate, all anionic surfactants);

sulfonates of naphthalene and alkyl naphthalene (such as sodium alkyl naphthalene sulfonate, sodium n-butyl naphthalene sulfonate, and sodium diisopropyl naphthalene sulfonate, all anionic surfactants); sulfonates of petroleum (such as sodium petroleum sulfonate, an anionic surfactant);

sulfoccinamates (such as disodium N-octadecyl sulfosuccinamate, tetrasodium N-(1,2-dicarboxyethyl)-N-octadecylsulfosuccinamate, and di-ammonium N-octadecylsulfosuccinamate, all anionic surfactants);

sulfosuccinates and derivatives (such as dioctyl sulfosuccinate sodium salt, disodium lauryl sulfosuccinate, and disodium mono-oleamido PEG-2 sulfosuccinate, all anionic surfactants);

taurates (such as sodium-N-methyl-N-oleyl-taurate, an anionic surfactant);

thio and mercapto derivatives (such as polyoxyethylene thioethers and ethoxylated dodecylmercaptan, nonionic surfactants);

and tridecyl and dodecyl benzene sulfonic acids (such as modified dodecyl benzene sulfonic acid, an anionic surfactant). Further examples are given in <u>McCutcheon's Emulsifiers & Detergents</u> (McCutcheon Division, MC Publishing Co., New Jersey (1994)).

Further non-limiting examples of
surfactants useful herein include the conventional
$C_{11}-C_{18}$ alkylbenzene sulphonates ("LAS"); the $C_{10}-C_{18}$
secondary (2,3) alkyl sulphates of the formula
$CH_3(CH_2)_x (CHOSO_3^-M^+)CH_3$ and $CH_3(CH_2)_y (CHOSO_3^-M^+)CH_2CH_3$
where x and (y+1) are integers of at least about 7,
preferably at least about 9, and M is a water-
solubilizing cation, especially sodium; unsaturated
sulphates such as oleyl sulphate; $C_{10}-C_{18}$ alkyl alkoxy
carboxylates (especially the EO 1-7
ethoxycarboxylates); the $C_{10}-C_{18}$ glycerol ethers; the
$C_{10}-C_{18}$ alkyl polyglycosides and their corresponding
sulphated polyglycosides; and $C_{12}-C_{18}$ alpha-sulphonated
fatty acid esters. If desired, the conventional
nonionic and amphoteric surfactants such as the $C_{12}-C_{18}$
alkyl ethoxylates ("AE") including the so-called
narrow peaked alkyl ethoxylates and C_6-C_{12} alkyl
phenol alkoxylates (especially ethoxylates and mixed
ethoxy/propoxy), $C_{12}-C_{18}$ betaines and sulphobetaines
("sultaines"), $C_{10}-C_{18}$ amine oxides, and the like, can
also be used. The $C_{10}-C_{18}$ N-alkyl polyhydroxy fatty
acid amides can also be used. Typical examples
include the $C_{12}-C_{18}$ N-methylglucamides. See WO
9,206,154. Other sugar-derived surfactants include
the N-alkoxy polyhydroxy fatty acid amides, such as
$C_{10}-C_{18}$ N-(3-methoxypropyl) glucamide. $C_{10}-C_{20}$
conventional soaps or branched-chain $C_{10}-C_{16}$ soaps may
also be used.

Other anionic surfactants can also be used,
including salts (including, for example, sodium
potassium, ammonium, and substituted ammonium salts
such a mono-, di- and triethanolamine salts) of soap;
C_9-C_{20} linear alkylbenzenesulphonates; C_8-C_{22} primary
or secondary alkanesulphonates; C_8-C_{24}

olefinsulphonates; sulphonated polycarboxylic acids;
alkyl glycerol sulphonates; fatty acyl glycerol
sulphonates; fatty oleyl glycerol sulphates; alkyl
phenol ethylene oxide ether sulphates; paraffin
sulphonates; alkyl phosphates; isothionates such as
the acyl isothionates; N-acyl taurates; fatty acid
amides of methyl tauride; alkyl succinamates and
sulphosuccinates; monoesters of sulphosuccinate
(especially saturated and unsaturated C_{12}-C_{18}
monoesters); diesters of sulphosuccinate (especially
saturated and unsaturated C_6-C_{14} diesters); N-acyl
sarcosinates; sulphates of alkylpolysaccharides such
as the sulphates of alkylpolyglucoside; branched
primary alkyl sulphates; alkyl polyethoxy
carboxylates such as those of the formula $RO(CH_2CH_2O)_k$
CH_2COO^--M^+ wherein R is a C_8-C_{22} alkyl, k is an integer
from 0 to 10, and M is a soluble salt-forming cation;
and fatty acids esterified with isethionic acid and
neutralized with sodium hydroxide. Further examples
are given in Surface Active Agents and Detergents
(Vol. I and II by Schwartz, Perry and Berch).

Further exemplary surfactants useful in the
present invention include polyoxyethylene(10)-
isooctylphenyl ether (Triton®X-100); sodium dodecyl
sulfate (SDS), Sarcosyl™, polysorbate 80 (Tween® 80),
Tween® 85, polyoxyethylene (10) cetyl ether (Ceteth™
10; Brij® 56), polyethylene glycol, polypropylene
glycol, dansyl-polymyxin and the like. In an
especially preferred embodiment, the detergent is
N-lauroylsarcosine. In another especially preferred
embodiment, the detergent is sodium lauryl sulfate.

The resulting homogenate is then admixed
with a water-immiscible organic solvent under
conditions and for a time appropriate to form an

aqueous phase and an organic phase. The use of a
water-immiscible organic solvent to form two phases
is well known to workers of ordinary skill in the
art, as are the reaction conditions and times. For
example, an ice-cold (approximately zero °C) solution
of a water-immiscible organic solvent can be admixed
into the homogenate by rapid shaking for a relatively
short period of time, from about 10 to about 60
seconds, preferably about 15 seconds.

A water-immiscible organic solvent is one
which, when admixed with deionized water, forms two
liquid phases separated by a phase boundary or a
meniscus. Exemplary water-immiscible organic
solvents are chloroform, carbon tetrachloride,
isoamyl alcohol, toluene, diethyl ether, 1-bromo-3-
chloropropane, and combinations thereof. In general,
organic solvents with low polarity can be used as
water-immiscible organic solvents. Other such water-
insoluble organic solvents are well known to workers
of ordinary skill in the art.

The resolution of the aqueous phase and the
organic phase can be facilitated by centrifugation or
ultracentrifugation, although the two phases will
resolve if the sample is left undisturbed for a
period of time until the two phases are visually
separate. The aqueous phase is generally found above
the organic phase, and the aqueous phase can be
removed by techniques well known to workers of
ordinary skill in the art. For example, the aqueous
phase can be removed by pipetting or micropipetting,
or by aspiration, or similar techniques. In the
event that the aqueous phase is found below the
organic phase, the aqueous phase can be recovered by
simply removing the upper organic phase, discarding

the upper organic phase, and retaining the aqueous
phase for further analysis. These techniques are
well known to workers of ordinary skill in the art.

The aqueous phase is then contacted with a
C_1-C_4 lower alcohol under conditions and for a time to
form a precipitated RNA. The use of a C_1-C_4 lower
alcohol to precipitate RNA is well known to workers
of ordinary skill in the art, as are the reaction
conditions and times. For example, an ice-cold
(approximately 0 °C) C_1-C_4 lower alcohol can be
admixed with the aqueous phase by inversion for a
relatively short period of time, from about 10 to
about 60 seconds, preferably about 15 seconds, until
a precipitate is formed. Preferred C_1-C_4 lower
alcohols include ethanol, methanol, isopropyl
alcohol, 2-propanol and 2-methyl-2-propanol.
Isopropyl alcohol (isopropanol) is especially
preferred. Other such C_1-C_4 lower alcohols are well
known to workers of ordinary skill in the art.

The precipitated RNA is then recovered as
by centrifugation and decanting of the aqueous phase,
according to techniques that are well known to
workers of ordinary skill in the art. For example,
the precipitated RNA can be recovered by
ultracentrifugation or microcentrifugation, and the
resulting supernate discarded by aspiration or
pouring. The precipitated RNA can comprise total
RNA. The RNA or total RNA is then ready for further
manipulation.

In a further embodiment, the present
invention contemplates a method of isolating total
RNA from a Gram-positive bacterium. Gram-positive
bacteria are further exemplified elsewhere herein,
and can include medically important Gram-positive

bacteria that are causative agents for diseases in
humans and other animals. In this embodiment, the
Gram-positive bacterium is contacted with an
admixture of (i) a mono-phasic solution of phenol and
guanidine isothiocyanate and (ii) a lysis buffer
under conditions and for a time sufficient to form a
homogenate, as discussed elsewhere herein. Next, the
homogenate is admixed with a water-immiscible organic
solvent under conditions and for a time sufficient to
form an aqueous phase and an organic phase, as
discussed elsewhere herein. The aqueous phase is
then contacted with a C_1-C_4 lower alcohol under
conditions and for a time to form a precipitated RNA,
as discussed elsewhere herein. The precipitated RNA
is then recovered, as by centrifugation and decanting
of the aqueous phase, as discussed elsewhere herein.

In another aspect, the invention
contemplates a composition of matter comprising an
admixture of (i) a mono-phasic solution of phenol and
guanidine isothiocyanate and (ii) a lysis buffer.
The components of this admixture are discussed more
extensively elsewhere herein. The composition of
matter is useful for isolating RNA or total RNA from
a biological specimen, according to methods set forth
herein or other such methods.

In another embodiment, the present
invention comprises a kit comprising an admixture of
(i) a mono-phasic solution of phenol and guanidine
isothiocyanate and (ii) a lysis buffer. In a further
embodiment, the present invention comprises a kit
comprising (i) a mono-phasic solution of phenol and
guanidine isothiocyanate and (ii) a lysis buffer.

The above kits optionally include
instructions for isolating RNA from a biological

specimen. Alternatively, the above kits optionally
include instructions for isolating total RNA from a
biological specimen. In preferred embodiments, the
biological specimen is a bacterium. In especially
preferred embodiments, the bacterium is a Gram-
positive bacterium. The kit optionally includes
vials, tubes, containers, packaging, inserts, and the
like, as is well known by workers of ordinary skill
in the art. For example, the admixtures and
solutions of a kit of the invention are placed in
appropriate vials, tubes or similar containers for
each of manufacture, use, storage and shipment.

Using the kits, compositions and methods of
the invention, RNA and/or total RNA can be isolated
from biological specimens, including microorganisms
such as bacterial species such as *Escherichia coli,*
Salmonella enterica, Klebsiella pneumoniae,
Enterobacter cloacae, Citrobacter freundii,
Morganella morganii, Hafnia alvei, Serratia
mercescens, Pseudomonas aeruginosa, Helicobacter
pylori, Staphylococcus aureus and *Streptoccus*
pneumoniae, fungal species (including yeast), viral
species, bacteriophages, other microscopic organisms,
tissue culture samples from eukaryotic or prokaryotic
species, laboratory cultures of eukaryotic or
prokaryotic species, clinical cultures and the like.
Microorganisms include viruses (both extracellular
and intracellular), bacteria (both eubacteria and
archaebacteria), bacteriophages, fungi (including
yeast), blood-transmitted parasites, and protozoa.
Exemplary viruses include acquired immunodeficiency
(HIV) virus, hepatitis A, B and C viruses, sinbis
virus, cytomegalovirus, vesicular stomatitis virus,
herpes simplex viruses, e.g. types I and II, human T-

lymphotropic retroviruses, HTLV-III, lymphadenopathy virus LAV/IDAV, parvovirus, transfusion-transmitted (TT) virus, Epstein-Barr virus, and others known to the art. Bacteriophages include .PHI.X174, .PHI.6, .lambda., R17, T.sub.4, and T.sub.2. Exemplary bacteria include *P. aeruginosa, S. aureus, S. epidermidis, L. monocytogenes, E. coli, K. pneumonia* and *S. marcescens*. Surprisingly, RNA thus isolated is typically free from DNA contamination, as assayed by gel electrophoresis or PCR. Genomic DNA, if detected, is present in reduced amounts relative to other methods of isolating RNA or total RNA.

Medically or clinically relevant bacteria are often categorized by their ability to retain a colored dye, using the Gram stain technique well known in the art. Medically important Gram-positive bacilli that can be used in the methods of the present invention include *Actinomyces spp., Bifidobacterium spp., Clostiridim spp., Bacillus spp., Listeria spp., Nocardia spp., Corynebacterium spp.* and *Propionibacterium spp.* Medically important Gram-positive cocci that can be used in the methods of the present invention include *Staphylococcus spp., Streptococcus spp.,* and *Peptostreptococcus spp.* Medically important Gram-negative bacilli that can be used in the methods of the present invention include *Aceinetobacter spp., Actinobacillus spp., Bordetella spp., Brucella spp., Campylobacter spp., Capnocytophaga spp., Cardiobacterium spp., Eikenella spp., Francisella spp., Haemophilus spp., Helicobacter spp., Kingella spp., Legionella spp., Morganella spp., Pasteurella spp., Citrobacter spp., Enterobacter spp., Escherichia spp., Klebsiella spp., Proteus spp., Salmonella spp., Shigella spp.,*

Serratia spp., Yersinia spp., Pseudomonas spp., Flavobacterium spp., Xanthomonas spp., Burkholderia spp., Aeromonas spp., Plesiomonas spp., and *Vibrio spp.* Medically important Gram-negative cocci that can be used in the methods of the present invention include *Neisseria spp., Moraxella spp.,* and *Veillonella spp.*

EXAMPLES

Example 1. Preparation of RNA from Clinical Samples

An overnight (about 16 hours) culture of a clinical microbial sample was diluted in a ratio of 1 to 100 in fresh growth medium and grown to a desired optical density (OD). Approximately 1.5 milliliters of this culture were transferred to a pre-chilled microfuge tube and spun in a tabletop centrifuge at top speed for approximately 2 minutes at 4° C. The supernatant was removed, and the resulting microbial pellet was suspended in 800 microliters of Trizol® and 200 microliters of lysis buffer. The lysis buffer was preheated to 95° C in a water bath for 15 minutes.

The lysis buffer was composed of 190 millimolar EDTA, 10 millimolar EGTA, 3.0% N-lauroylsarcosine, and 1.0 molar sodium acetate, and was prepared as follows. The ingredients were dissolved in water, with constant stirring and heating. The resulting solution was allowed to stand at 60° C for 30 minutes and at room temperature (approximately 20° C) for approximately 16 hours, or overnight. Afterwards, the supernatant portion was carefully transferred to a fresh bottle without disturbing any precipitate.

The microbial pellet was mixed with the Trizol®/lysis buffer admixture by rapid pipetting.

The resulting homogenate was incubated at 95° C for 4 minutes. The homogenate was transferred to room temperature (approximately 20° C) for 5 minutes. Next, 200 microliters of ice cold chloroform was added, and mixed by rapid shaking for 15 seconds. The resulting admixture was spun at 8000 revolutions per minute for 15 minutes at 4° C. Then, 400 microliters of the colorless, upper aqueous phase was carefully removed to a fresh microfuge tube. Next, 400 microliters of ice-cold isopropanol were added and mixed with the aqueous phase by inversion.

A precipitate was allowed to form at room temperature (approximately 20° C) for 10 minutes. The precipitate was spun down at 8000 revolutions per minute for 10 minutes at 4° C. The supernatant was then discarded and 1 milliliter of ice cold 75 percent ethanol was added and mixed by inversion. The mixture was then spun at 5000 revolutions per minute for 5 minutes at 4° C. After removing the supernatant, the pellet was air dried for approximately 5 minutes, with care taken not to dry the pellet completely as doing so decreased the solubility of the resulting RNA. The pellet was then resuspended in 50 microliters of RNase-free water or TE buffer (10 millimolar Tris − 1 millimolar EDTA). The RNA concentration was determined with a spectrophotometer, and the purity and yield of the process was determined by analyzing 1 microgram of RNA on a 1 percent non-denaturing agarose gel according to techniques well known to those of ordinary skill in the art. The RNA was then stored at −80 °C until further use.

RT-PCR was performed using 250 nanograms of RNA isolated as above with the OneStep™ RT-PCR Kit

(Qiagen; Valencia, CA, USA) according to the
manufacturer's instructions. This kit contains a
mixture of enzymes, in particular the Omniscript™ and
Sensiscript™ reverse transcriptases and the HotStar™
Taq DNA polymerase. Forward and reverse primers were
used at 0.5 micromolar concentrations and the final
concentration of each dNTP was 400 micromolar. The
actual RT-PCR reaction was conducted as follows:
reverse transcription reaction for 30 minutes at 50
°C; initial PCR activation for 15 minutes at 95° C;
and three-step cycling (30 to 60 seconds at 94° C for
denaturation, followed by 30 seconds at 50° C for
annealing, and 1 minute at 72° C for extension). The
final extension was carried out at 72° C for 10
minutes. Reaction products were analyzed on 1
percent agarose gels stained with ethidium bromide
according to techniques well known to those of
ordinary skill in the art.

Primer extension analysis was used to
determine the start site of transcription and also
the level of gene expression. Total RNA was isolated
as set forth above. Primers were annealed to 20
micrograms of total RNA at 50° C and primer extension
was performed using 100 units of MuLV reverse
transcriptase (Perkin-Elmer, Norwalk, CT, USA) as
previously described [Reisbig et al, *J. Antimicrobial
Chemotherapy*, 51:1141-1151 (2003)].

Example 2. Preparation of RNA Samples
            using Hot Phenol Method
            On the first day of preparation, an
overnight (about 16 hours) culture of a clinical
isolate was diluted in a ratio of 1 to 20 in Mueller-
Hinton Broth, for a total volume of 100 milliliters.

The culture was incubated at 37° C until the desired
OD was obtained. Afterwards, 4 milliliters of the
culture were removed and added to a test tube
containing 1 milliliter of lysis buffer prewarmed to
95° C. The resulting mixture was incubated at 95° C
for 5 minutes. The hot lysate was then poured into a
flask containing acidic phenol (pH value of about
4.8) pre-warmed to 68° C and vigorously mixed for 5
minutes in a shaker water bath set to 68° C. The
resulting hot phenol mixture was poured into a pre-
cooled tube and chilled on ice for 5 minutes.

    The tube was then centrifuged at 3100
revolutions per minute using a Sorvall H1000B rotor
for 10 minutes at 2° C. The aqueous phase was
removed to a new pre-chilled tube, and an equal
volume of acidic phenol was added and mixed
vigorously. This mixture was then centrifuged at
3100 revolutions per minute as before, and the
aqueous phase removed to another pre-chilled tube.
To that aqueous phase, 0.25 volumes of 10 molar LiCl
was added and mixed well. A precipitate was allowed
to form for 16 hours (or overnight) at 4° C.

    On the second day of preparation, the
sample was centrifuged at 3100 revolutions per minute
for 10 minutes at 2° C, as before, and the RNA pellet
was washed with 2 molar LiCl. The resulting RNA
pellet was then dissolved in 400 microliters of DEPC-
H_2O and transferred to a microfuge tube. Then, 3
molar sodium acetate (pH 5.2) was added to a final
concentration of 0.3 molar (approximately 40
microliters), and 2.5 volumes of 100 percent ethanol
was added. The RNA was precipitated for at least 2
hours at -20 °C, or for 30 minutes on dry ice. The
RNA was pelleted by spinning in a microfuge at top

speed for 10 minutes. The resulting RNA pellet was
washed once with 70 percent ethanol, and the RNA
pellet was resuspended in 200 microliters 1 x TE
buffer (pH 8.0). The RNA concentration was
determined with a spectrophotometer. A one microgram
sample was run on a 1 percent agarose gel to
determine yield and purity of the sample according to
techniques that are well known to those of ordinary
skill in the art. The RNA was stored at -80° C until
further use.

Example 3.Preparation of Total RNA

An overnight (about 16 hours) culture of a
bacterial specimen is diluted 1:100 and grown to a
desired optical density (for example, for three
hours). A 1.5 milliliter sample of the culture is
transferred to a pre-chilled microfuge tube and is
spun at top speed for 2 minutes at 4° C. The
supernatant is removed and the resulting pellet is
suspended in 200 microliters of lysis buffer (the
lysis buffer is pre-heated to 95° C for 10 to 15
minutes). This suspension is incubated at 95° C for
4 minutes. Next, 1 milliliter of Trizol® is added.

The resulting homogenate is incubated at
room temperature for 5 minutes. Then, 200
microliters of ice-cold (approximately zero degrees
C) chloroform is added, and is mixed by rapid shaking
for 15 seconds. The resulting mixture is spun at
8000 revolutions per minute for 15 minutes at 4° C.
Thereafter, 400 microliters of the colorless, upper
aqueous phase is removed to a fresh tube (the aqueous
phase is removed by pipetting from the top down).

To this aqueous phase 400 microliters of
ice-cold (approximately zero degrees C) isopropanol

is added and mixed by inversion. The resulting total
RNA is precipitated at room temperature
(approximately 20° C) for 10 minutes. The
precipitated RNA is recovered by spinning at 8000
revolutions per minute for 10 minutes at 4° C.

The supernatant is then discarded and 1
milliliter of ice cold (approximately zero degrees C)
75 percent ethanol is added and mixed by inversion.
The sample is spun at 5000 revolutions per minute for
5 minutes at 4° C, and the supernatant again
discarded. The resulting total RNA pellet is air-
dried for 5 minutes, taking care not to completely
dry the pellet, as doing so will decrease the
solubility of the resulting total RNA. The air-dried
total RNA is then resuspended in 50 microliters of
RNase-free water or TE buffer.

The foregoing description and the examples
are intended as illustrative and are not to be taken
as limiting. Still other variations within the
spirit and scope of this invention are possible and
will readily present themselves to those skilled in
the art.

Each of the patents and articles cited
herein is incorporated by reference. The use of the
article "a" or "an" is intended to include one or
more.